CONFIDENTIAL
INVENTION DISCLOSURE

The invention herein described was made during the course of my employment and is being submitted in pursuance of the terms of the Employee Confidentiality Agreement.
(*Please use ink and attach extra sheets of paper if needed.)*

1. Title of Invention:

Mutated Epidermal Growth Factor Receptor as a selectable cell surface marker.

2. Brief Description of Invention:

Describe the invention, include any drawings, chemical structures, equipment designs, process steps. Experimental data may be included.

The present invention provides a method to use mutated versions of the epidermal growth factor receptor (EGFR) as a selectable cell surface marker. The EGFR was mutated in the extracellular as well as the intracellar domain in such a way that neither ligand binding nor signal transduction through this receptor occurs (see Fig.1 mutated EGFRII). This will therefore render the molecule inert. Thus, introduction of this mutated EGFR in eukaryotic cells e.g. cells of hematopoietic or others should provide a safe means to identify and select mutated EGFR expressing cells with an antibody directed against the mutated EGFR. Other molecules that were similarly rendered inert by mutating the intracellular and extracellular domain include Muscle specific receptor receptor tyrosine kinase (MuSK) or the γ-amino butyric acid receptor $_B$1/2 (GABAR$_B$1/2). These mutated molecules can be also used as selectable marker.

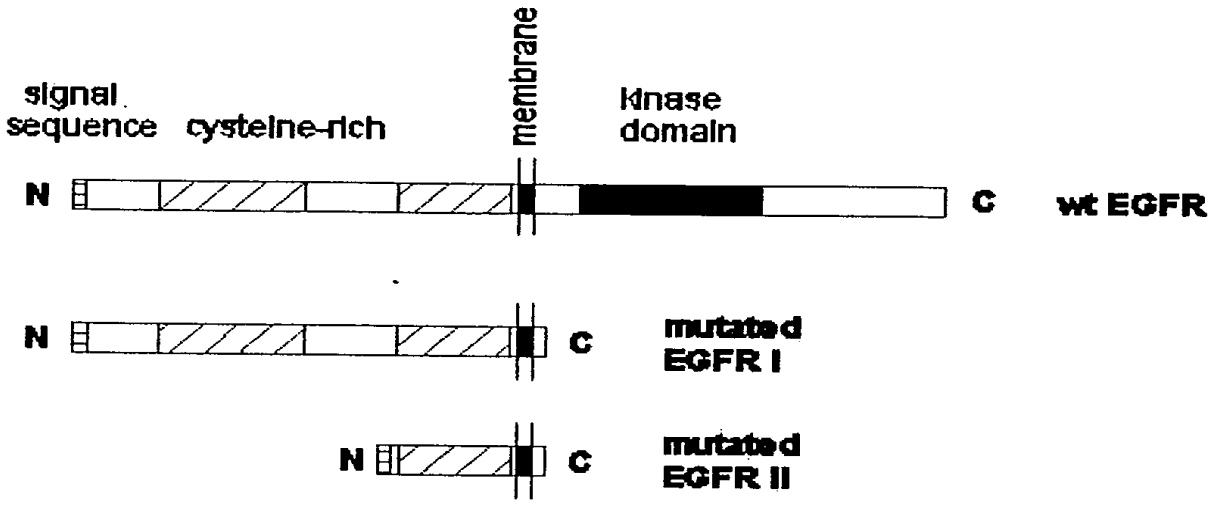

Fig.1.: This figure shows the mutations that were introduced in the EGFR to render the molecule inert. EGFRI has a mutation/deletion in the intracellular domain only. EGFRII has an additional mutation/deletion in the extracellular domain.

3. Novel Aspects:

Describe novel aspects of invention, i.e., how it is new and different.

The invention is new in that human cell surface molecules e.g. EGFR, MuSK, $GABAR_B1/2$ are used, which have not been used in the past for this kind of application.
Previously, cell surface molecules that were used as cell surface markers were mutated in their intracellular domain to avoid signaling of these newly introduced molecules when they would bind to their ligand. However, upon binding of to their ligands these intracellularly mutated molecules could potentially still heterodimerize with endogenous receptors and could therefore result in a dominant negative effect (see Fig.1/mutated EGFRI. This molecule has been previously described in patent WO 93/05148 as a mutant EGFR that is called HERCD-533 and that is devoid of signaling activity.). To avoid this problem we also mutated parts of the extracellular domain to prevent ligand binding (see Fig. 1 mutated EGFRII). However, extracellular mutations were done in such a way that antibody binding to the extracellular domain can still occur and therefore effective identification and selection of marker gene carrying cells is possible. This therefore adds another new safety feature to the usage of these molecules as cell surface markers.

4. Pertinent References of Which You are Aware:

List literature (including abstracts), patent applications, patents, and presentations, with respect to efforts to deal with the kind of problem your invention is designed to solve.

O. Kashles, Y. Yarden, R. Fischer, A. Ullrich and J. Schlessinger MCB 1991, 11: 1454-1463, A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization.

C. R. Lin, W. S. Chen, W. Kruiger, L. S. Stolarsky, W. Weber, R. M. Evans, I. M. Verma, G. N. Gill, M. G. Rosenfeld. Science 1984, 224: 843-847: Expression Cloning of Human EGF Receptor Complementary DNA: Gene Amplification and Three Related Messenger RNA in A431 Cells.

Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells.

K. Kaupmann, K. Huggel, J. Heid, P. J. Fior, S. Bischoff, S. J. Mickel, G. McMaster, C. Angst, H. Bittiger, W. Froestl, B. Bettler. Nature 1997, 386: 239-246. Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors.

K. Kaupmann, B. Malitscheck, V. Schuler, J. Heid, W. Froestl, P. Beck, J. Mosbacher, S. Bischoff, A. Kulik, R. Shigemoto, A. Karschin, B. Bettler. Nature 1998, 396: 683-687. $GABA_B$-receptor subtypes assemble into functional heteromeric complexes.

D.M. Valenzuela, T. N. Stitt, P. S. DiStefano, E. Rojas, K. Mattsson, D. L. Compton, L. Nunez, J. S. Park, J. L. Stark, D. R. Gies, S. Thomas, M. M. LeBeau, A. A. Fernald, N. G. Copeland, N. A. Jenkins, S. J. Burden, D. J. Glass, G. Yancopoulos. Neuron 1995, 15: 573-584. Receptor tyrosine kinase specific for the skeletal muscle lineage: Expression in embryonic muscle, at the neuromuscular junction, and after injury.

Patents: WO93/05148, PCT/EP94/02687

5. Utility of Invention:

Describe any other possible applications of the invention beyond the intended primary application. Describe any commercial aspects of the invention.

The selectable marker will be part of a product, either cell e.g. hemopoietic stem cell or vector system. Thus the commercial value will depend on the product the selectable marker is sold with.

6. Date of Invention:

7. Disclosure Outside of SyStemix:

List places, dates and names of persons or companies to whom disclosed (or planned to be disclosed) outside of SyStemix (regardless of the existence of a nondisclosure agreement).

N/A

8. Documentation:

A notebook reference and location of notebook.

See attached documents

9. Program or Contract:

Was invention made during the course of your work on a specific program or contract?

Yes: ___x_____ No: _____ Specific program or contract: ___34/05_____

10. Persons who Worked on Invention:

Susanne Pippig
Peter Chang
Gabor Veres

11. Person Preparing this Disclosure:

Signature: _____ Printed name: Susanne Pippig

Address: ___Systemix, Inc. 3155 Porter Drive, Palo Alto, CA 94304____

Date: _____ _____

12. Two Witnesses:

The invention was described to me by the above inventor(s); the description was examined and clearly understood.

Signature: _____ Printed name: ____Fernando Rock_____

Address: ___Systemix, Inc. 3155 Porter Drive, Palo Alto, CA 94304____

Date: __ _____

Signature: _____ Printed name: _____Ann Marie OFarrell____

Address: ___Systemix, Inc. 3155 Porter Drive, Palo Alto, CA 94304____

Date: __ _____

Purpose: clone hEGFR deletition mutant

A) hEGFR PCR

Order primers from Operon

		Tm
hEGFR186F	cc T CTA gAg ATg CgA CCC TCC ggg Acg (XbaI)	~68.8°C
hEGFR2220R	g gA TAT C CT ACg TgC gCT TCC gAA CgA TgT g (EcoRV; thr654; stop codon)	~66.5°C

SEQUENCE INFORMATION FOR INVOICE B161247

hEGFR186F 5'- CCT CTA GAG ATG CGA CCC TCC GGT ACG -3'
31V5-244
27 mer
Unpurified product from 50 nmol synthesis scale
Yield = 15 OD or 60132 pmoles; approx 494 µg MW = 8220 D Trityl Group Removed ε_{260} = 249449 T_m = 74 C

hEGFR2220R 5'- GGA TAT CCT ACG TGC GCT TCC GAA CGA TGT G -3'
3191-245
31 mer
Unpurified product from 50 nmol synthesis scale
Yield = 21.5 OD or 73596 pmoles; approx 699 µg MW = 9497 D Trityl Group Removed ε_{260} = 292134 T_m = 71 C

1 µg/λ pNTK-EGFR	0.2λ	
hEGFR2220R (Tm71°C)	1	
hEGFR186F (Tm74°C)	1	
pfu buffer	5	
10mM dNTP	2	
ddH2O	38.8	
pfu	2	
	50λ	

5' 94°C
94°C 30"
66°C 60"
72°C 5'
(30λ)
72°C 10'
4°C

[redacted]

		10X
hEGFR PCR from above	2 λ	20
hEGFR2220R	1	10
hEGFR186F	1	10
pfu buffer	5	50
10mM dNTP	2	20
ddH2O	38	380
pfu	1	10
	50λ	50λ/tube

94°C 5' → 94°C 36" / 68°C 60" / 72°C 6' (10X) → 72°C 10' → 4°C soak

Load 12 λ from 450λ PCR product (pg66) gel isolation

[redacted] Gel Isol.

PCR Scr. Ligation → Tfm

Ask Susanne to take out plates & Innoculate

[redacted]

Take out late ⇒ satelite ~~dishes~~ colonies MUSK H2 & H3

~~Put into mini-prep~~

EcoRS + ~~Stu I~~

Innoculate #2 & 5

?

Qiagen maxiprep ⇒ pca yield?

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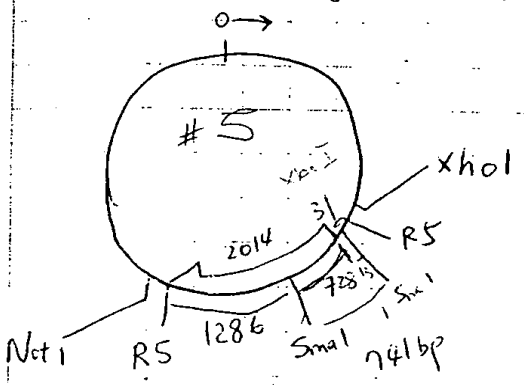
Peter Clay — Signed — Date

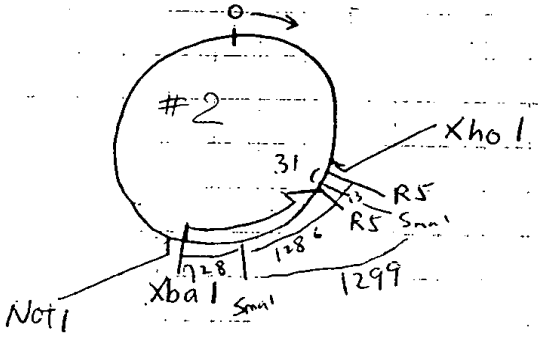
H2 — Signed — Date

H3 — Signed — Date

[redacted] Innoculate ... Also streak - check maxiprep (pg 66)

[redacted] Qiagen maxiprep again





EcoRS	2045, 2930 bp ✓	
Xba I	4944 bp ✓	
Sma I	741, 4257 bp	didn't cut ?

⇩

pCRScr-hEGFR #5

	728 ~~42~~ 16 bp	didn't cut
	4944	star activity ? ?
	1299, 3689 bp ✓	

⇩

pCRScr-hEGFR #2

) clone into pG1a-IRES-NGFR ∴ pG1a-hEGFR-IRES-NGFR

[redacted]

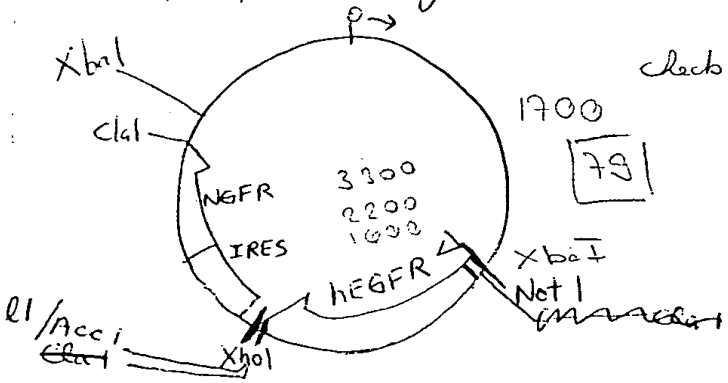
#1210 pG1a-IRES-NGFR (0.8 µg/λ)	12.5		pCRScr-hEGFR #2	25
Buffer H	10		Buffer H	10
Not I	5		Not I	5
Xho I	5		Xho I	5
TE	57.5		TE	55
	100 λ			100 λ

37°C 2:45

ethanol precipitate → gel isolation → Rapid DNA ligation kit : 1.5λ #1210
6.5λ pCRScr-hEGFR #2

↓ 4 o λ

Tfm XL 10 gold w/ 2λ lgt



check miniprep w/ Xba I

79

Continued on Page 72

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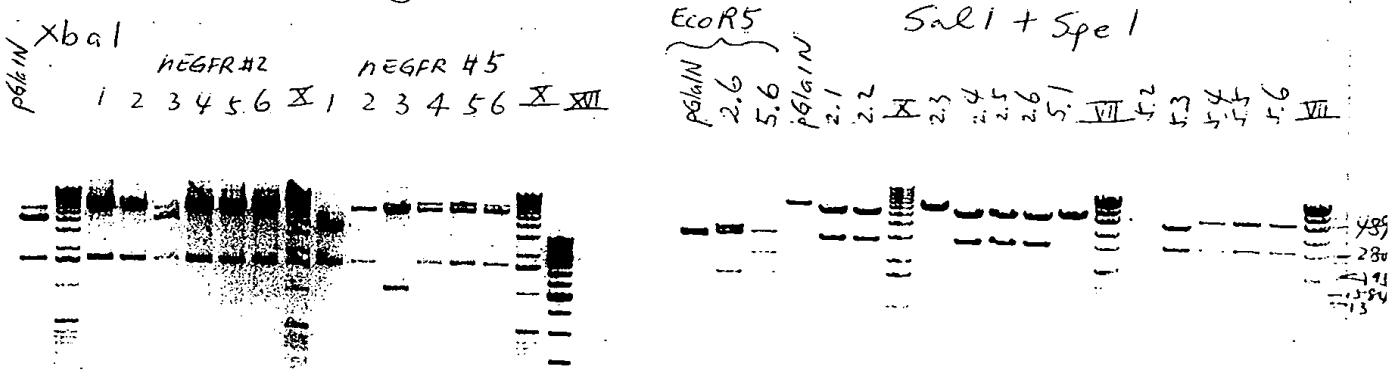
[redacted]
Date

[redacted]

	# colonies
#1210 control	12 plasmid
hEGFR 2△ insert control	0
hEGFR 5△ insert control	0
pGla-hEGFR 2△-IN	180
pGla-hEGFR 5△-IN	200

innoculate 6 colonies of each → 250 RPM 37°C 6:30 Am ~ 2 Pm



Xba I in primers methylation sensitive?

	pGla IN	pGla-hEGFR-IN
EcoR5	75.76.3016.3025	32.75.76.1750.3016.3330
Sal I + Spe I		2868, 5408

[redacted] Qiagen maxiprep

[81]

© clone pGla-hEGFR [redacted]

pGla	22 λ 0.452 μg/λ
H	10
Not I	5
Xho I	5
TE	78
	100 λ 37°C 10:10 Am – 12:40 Pm

dephosphorylation: add to above 100 λ reaction

20 λ CIP buffer
8 λ CIP
72 λ TE
200 λ 37°C 1 Pm ~

⇒ ethanol precipitate

Ⓓ clone pGla-IRES-hEGFR

eluted pGla (Not I + Xho I)	~~50~~ 40
buffer L	5
10mM dNTP	3
Klneow	2
	50 λ

30°C 15'
75°C 15'

Repeat above for pCRSu-hEGFR #2 frgmt (Not I + Xho I)

Continued on Page 73

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[redacted]
Date

© gel isolation → elute in ~~100~~ 80 λ ~100ng/λ

rapid DNA ligation

pGla (NotI+XhoI)	1
pCRScr-hEGFR #2 (NotI+XhoI) △	7
buffer 2	2
" 1	10
T4 DNA ligase	1
	21

1 4λ pGla NotI+XhoI, KInw (empty vector) fragmt A } gel
2 4λ " " " " fragmt B } isol
3 4λ pCRScr-hEGFR #2 △ " " "
4 4λ IRES XhoI+NcoI, KInw

Ⓓ rapid DNA ligation (3 way ligation)

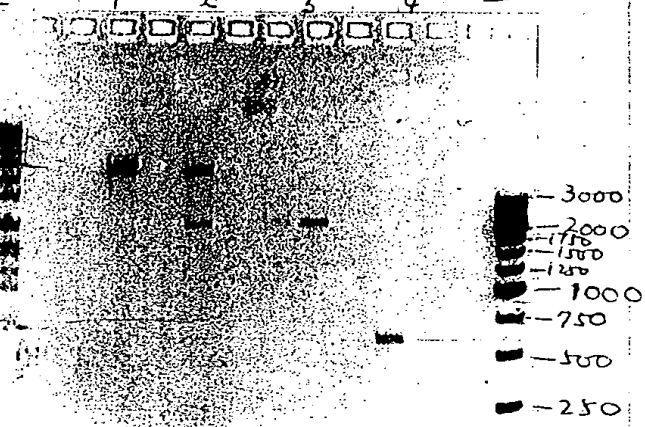
pGla (NotI+XhoI, KInw)		1
pCRScr-hEGFR (NotI+XhoI, KInw) △	4	9
IRES (XhoI+NcoI, KInw)	3	6
buffer 2	2	4
" 1	10	20
T4 DNA ligase		2
	21λ	42

25°C overnight pGly

[redacted]

tfm XL1 Blue (125λ) w/ 4λ ligation mixture → 30' ice
42°C 90"
ice 2'
37°C 250 RPM 30'
plate → 37°C shaker 1PM

4899 3639 2799 1953 1515 1164 992 710 492



pGly

[redacted]

innoculate 6 colonies from Ⓒ #1-6 } ~7 AM 4λ loaded 1% TEA agarose
12 Ⓓ 7-18 } →250 RPM 37°C →

	# colonies	check w/ SmaI	XbaI	NaeI+SpeI
pGla Ⓒ	120	1580, 3093	—	4673
pGla-hEGFR Ⓒ	1200	~~1997~~, 584, 749, 1015, 1324, 3893	—	2619, 4146
pGla Ⓓ	200	1580, 3093	—	
pGla-IRES-hEGFR Ⓓ	1000	—	308, 4309	

miniprep
RE 1 λ
buffer 1.2 λ
12 λ
37°C 90'

Continued on Page 74

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[redacted]
Date

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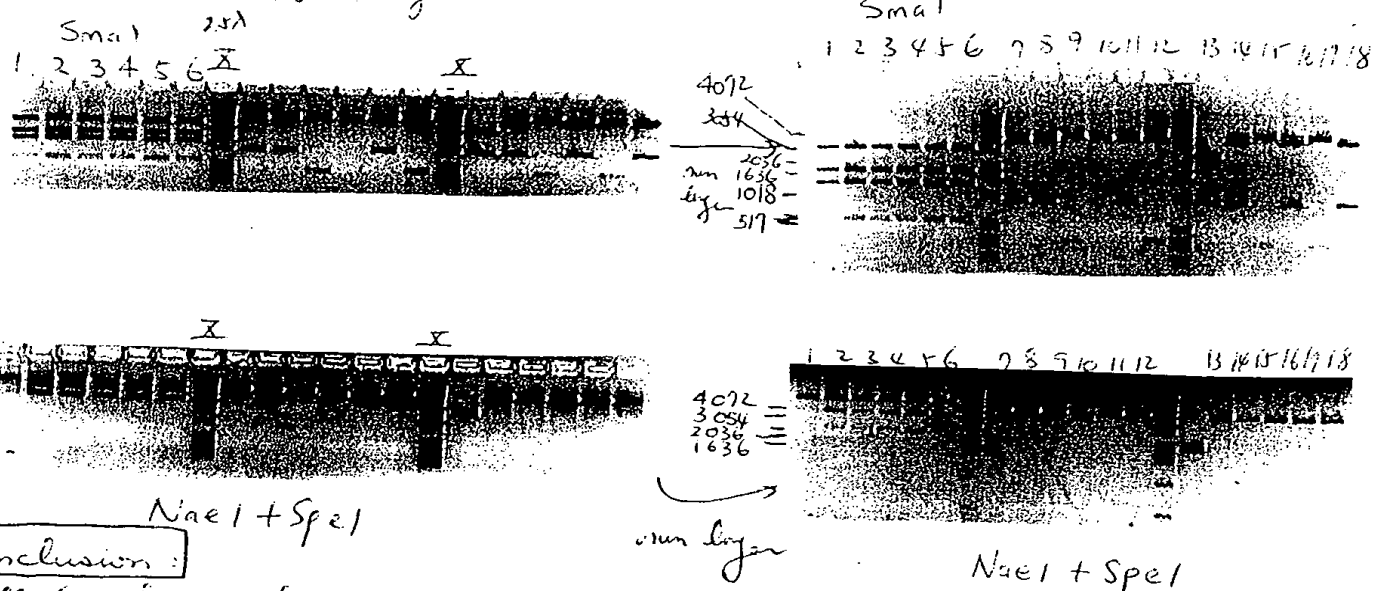
1% TEA agarose



conclusion:

① All 6 colonies of pGla-hEGFR give expected fragment sizes for SmaI & NaeI+SpeI digestion. NaeI + SpeI digestion is inefficient in buffer A : of SpeI (75-100%).

② 3 way ligation didn't work for pGla-IRES-hEGFR

Innoculate 500 mL LB+ 100 μg/mL Amp → Qiagen maxiprep for pGla-hEGFR
Thr 9-17-98 Harvest bacteria & store in -70°C
Fri 9-18-98 Qiagen maxiprep 1 column for 1L → wash 3x w/ 70% ethanol
get ~ 1500 λ (1μg/λ)

check with map

[redacted]

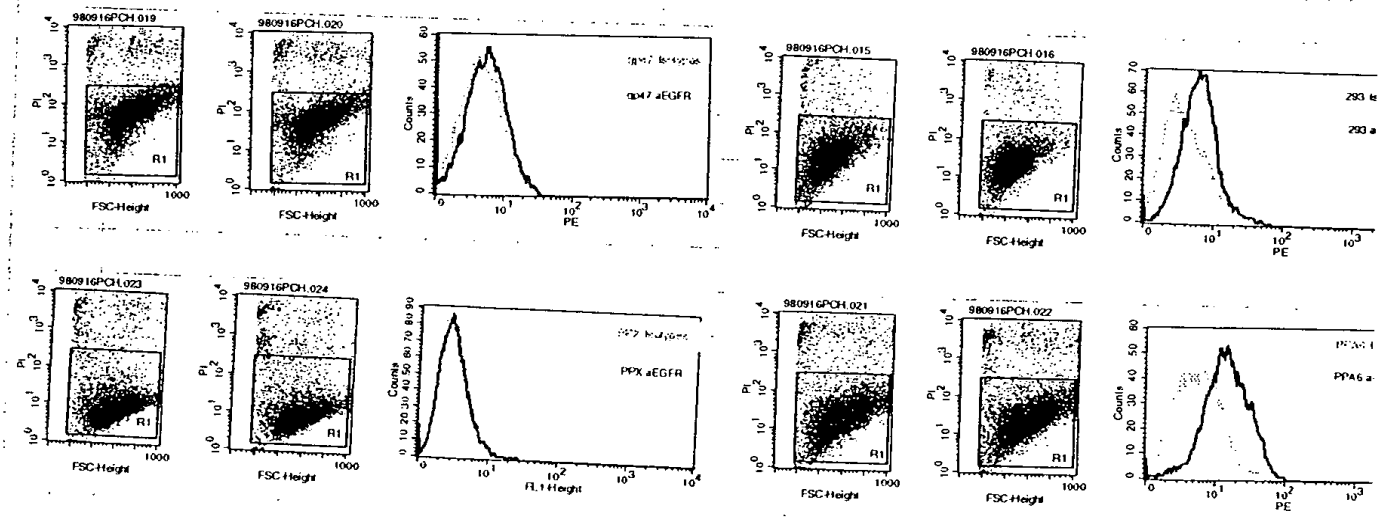
PROJECT hEGFRΔtfm Notebc . No. 1389 / 75

Continued From Page 75

[redacted] Seed 5-7x10^5 293 T's onto a 6 well plate well w/ 2mL
9:15 AM change/replace with fresh DMEM+10% FBS (2mL)
Add 10 λ 10 mM chloroquine to final conc. 50 μM
Put back into incubator ½ ~ 3 hrs
9:45 AM

				for 10 cm d
3 μg	pCIGL	(1.3 μg/λ)	2.5λ	15 μg
3 μg	pCIGP	(2 μg/λ)	1.5λ	15 μg
6 μg	pG1a-hEGFR-IRES-NGFR	(1μg/λ)	6	30 μg
	H_2O			λ
12.5λ	2M Ca			600λ
100 μl	in Falcon FACS tubes w/conical bottom			

add drop by drop equal volume of HEPES while vortexi
room temperature 15 ~ 20 min
10:05 AM vortex briefly before adding mixture to cells
mix "∞"
37°C
5:30 PM wash 1x & replace with 2mL fresh DMEM+10% FB
[redacted] Spin down cells from sup. Transfect PPA6 WT
Repeat spinoculation on PPA6 WT
Stain with αhEGFR*PE



Continued on Page 77

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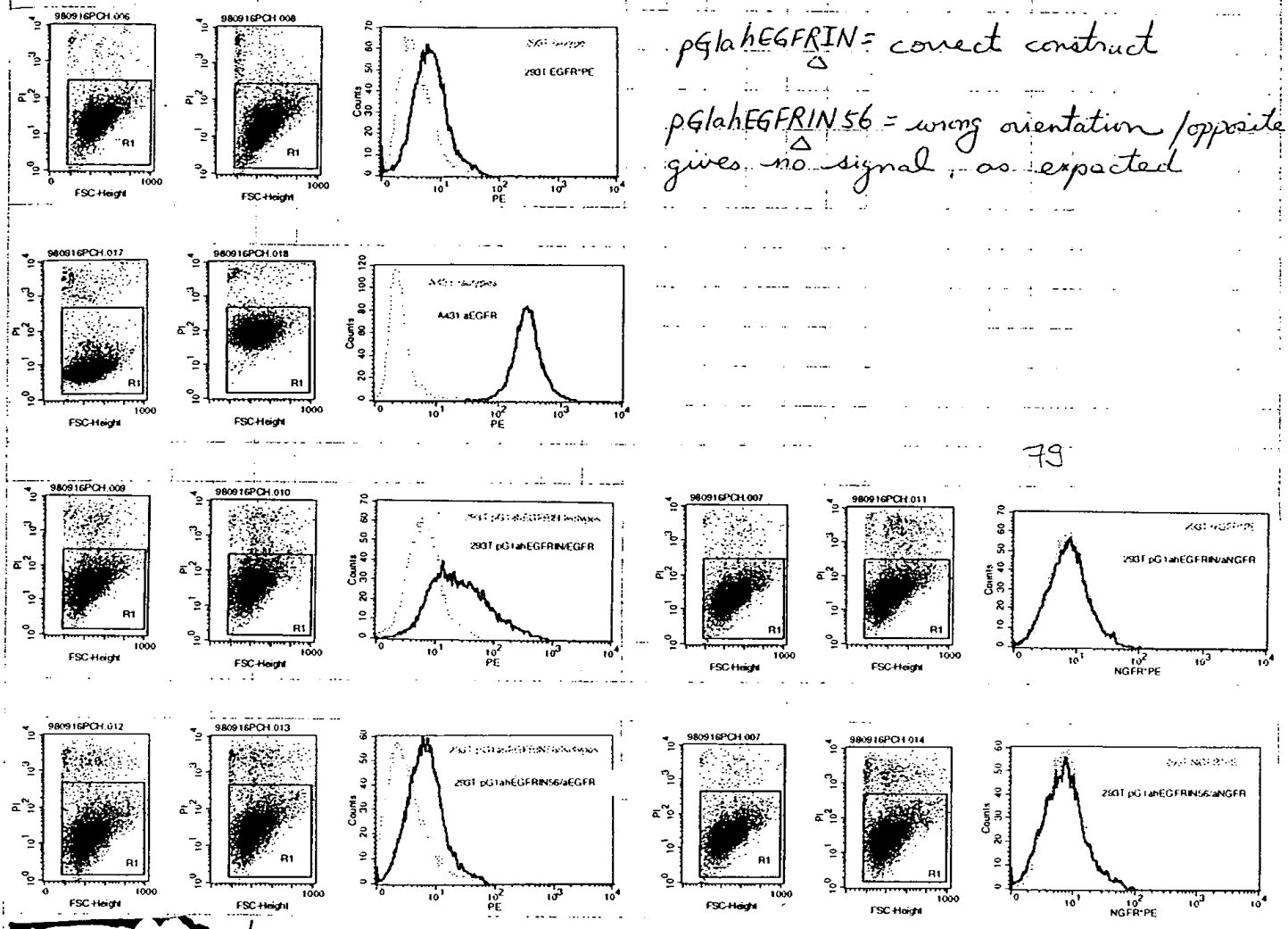
Signed [redacted] Date Signed [redacted] Date

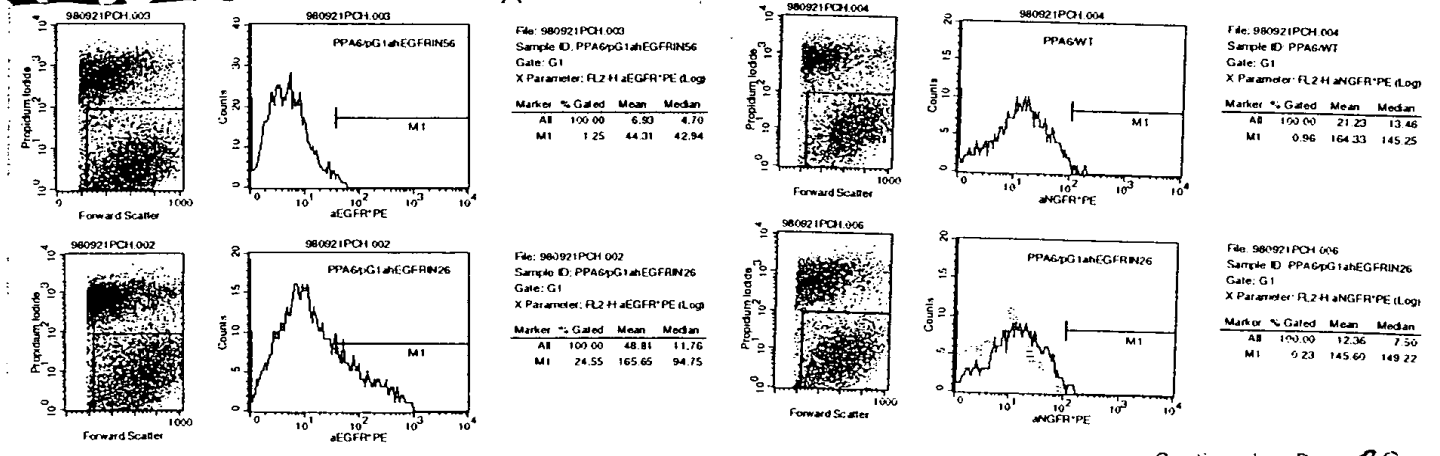
pGlahEGFRIN = correct construct

pGlahEGFRIN56 = wrong orientation / opposite gives no signal, as expected

79



[redacted] stain PPA6 for NGFR and EGFR



File: 980921PCH.003
Sample ID: PPA6/pG1ahEGFRIN56
Gate: G1
X Parameter: FL2-H aEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	6.93	4.70
M1	1.25	44.31	42.94

File: 980921PCH.004
Sample ID: PPA6/WT
Gate: G1
X Parameter: FL2-H aNGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	21.23	13.46
M1	0.96	164.33	145.25

File: 980921PCH.002
Sample ID: PPA6/pG1ahEGFRIN26
Gate: G1
X Parameter: FL2-H aEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	48.81	11.76
M1	24.55	165.65	94.75

File: 980921PCH.006
Sample ID: PPA6/pG1ahEGFRIN26
Gate: G1
X Parameter: FL2-H aNGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	12.36	7.50
M1	9.23	145.60	149.22

Continued on Page 79

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[redacted]
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[redacted] td {CEMSS WT} w/ supernatant pG1a hEGFRINGER from PPA6
{PBL's}

Abandoned ~ cells died

Repeat 1x more → cells stills died (from Jennifer Xu)

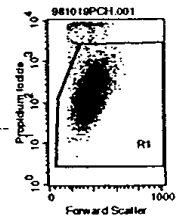
[redacted] Reseed 293T

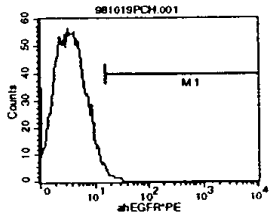
tfm

td PPA6 WT from Idein

FACS

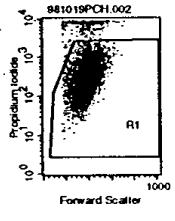
SORT for cells

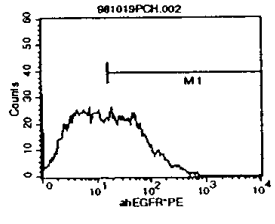




File: 981019PCH.001
Sample ID: PPA6/WT
Gate: G1
X Parameter: FL2-H ahEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	4.36	3.49
M1	1.09	21.34	19.63

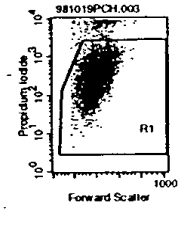


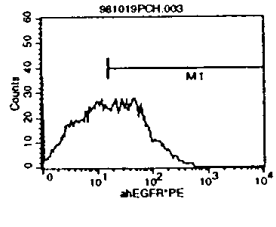


File: 981019PCH.002
Sample ID: PPA6/pG1a-hEGFR PCR-IN
Gate: G1
X Parameter: FL2-H ahEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	34.46	13.82
M1	46.90	66.27	42.17

79





File: 981019PCH.003
Sample ID: PPA6/pG1a-hEGFR PCR
Gate: G1
X Parameter: FL2-H ahEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	35.40	16.85
M1	51.75	62.03	42.55

81

Continued on Page

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[redacted]
Date

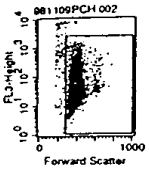
Purpose: Transduce PPA6 pGla-hEGFRΔ #79 81 onto CEMSS, CD34+

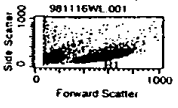
[redacted] Transduce CEMSS 2.5 hrs 32°C 3000 RPM

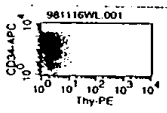
FACS

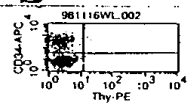
CEMSS

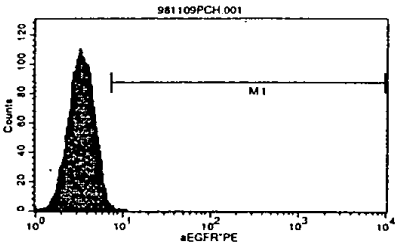
CD34+





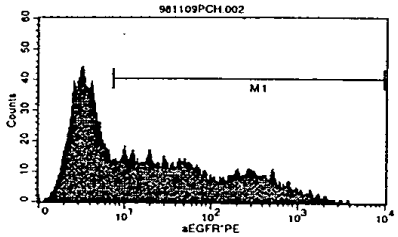






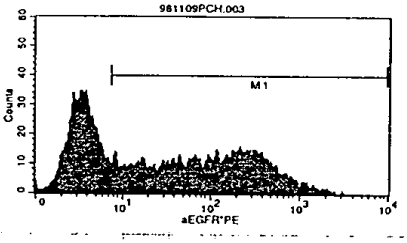
Sample ID: CEMSS/WT
Acquisition Date: 9-Nov-98
Gate: G1

Marker	% Gated	Mean	Median
All	100.00	3.66	3.37
M1	0.92	22.07	8.70



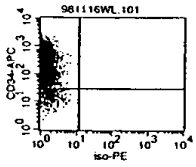
Sample ID: CEMSS/79
Acquisition Date: 9-Nov-98
Gate: G1

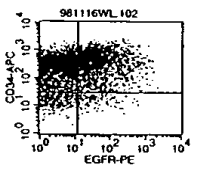
Marker	% Gated	Mean	Median
All	100.00	110.02	10.46
M1	55.68	194.71	52.33



Sample ID: CEMSS/81
Acquisition Date: 9-Nov-98
Gate: G1

Marker	% Gated	Mean	Median
All	100.00	136.53	20.17
M1	61.63	219.29	102.74



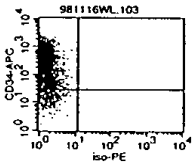


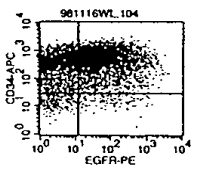
Sample ID: 79 EGFR-PE
Gated Events: 7735
Y Parameter: FL4-H CD34-APC (Log)

Quad	Events	% Gated	X Mean	Y Mean
UL	3817	49.35	4.70	322.87
UR	3302	42.69	82.96	479.71
LL	463	5.99	3.66	16.58
LR	153	1.98	71.34	16.77

Sample ID: 81 EGFR-PE
Gated Events: 7584
Y Parameter: FL4-H CD34-APC (Log)

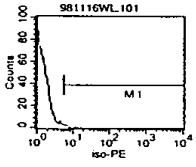
Quad	Events	% Gated	X Mean	Y Mean
UL	2951	38.91	4.63	443.58
UR	4201	55.39	155.35	594.91
LL	290	3.82	3.44	16.78
LR	142	1.87	178.19	17.67

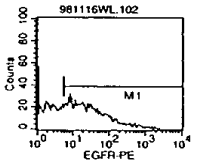




Sample ID: 79 iso-PE
Gated Events: 7527

Events	% Gated	Mean
7527	100.00	1.37
11	0.15	6.83



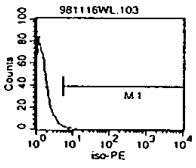


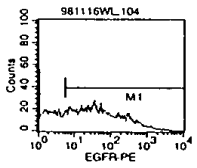
Sample ID: 79 EGFR-PE
Gated Events: 7735

Events	% Gated	Mean
7735	100.00	39.37
4919	63.59	60.44

Sample ID: 81 iso-PE
Gated Events: 7607

Events	% Gated	Mean
7607	100.00	1.41
23	0.30	6.80





Sample ID: 81 EGFR-PE
Gated Events: 7584

Events	% Gated	Mean
7584	100.00	91.32
5429	71.58	126.58

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Continued on Page 20

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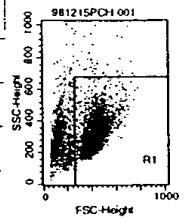
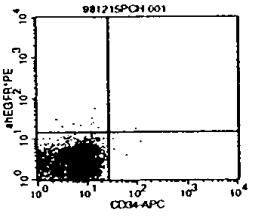
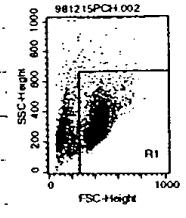
Signed [redacted] Date

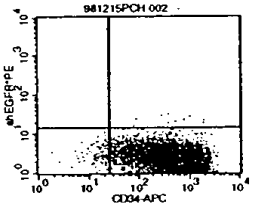




File: 981215PCH.001
Sample ID: CD34+/Isotypes
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

Quad	% Gated	X Mean	Y Mean
UL	0.17	10.76	27.25
UR	0.04	51.02	16.42
LL	99.67	6.94	2.97
LR	0.13	44.52	6.04

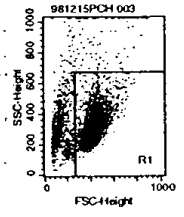


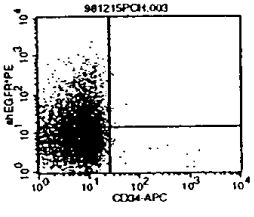


File: 981215PCH.002
Sample ID: CD34+/αCD34*APC
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

Quad	% Gated	X Mean	Y Mean
UL	0.00	***	***
UR	0.18	727.48	26.53
LL	1.41	17.60	3.62
LR	98.41	769.88	2.34

α CD34 APC
ISO PE

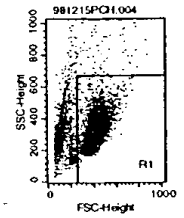


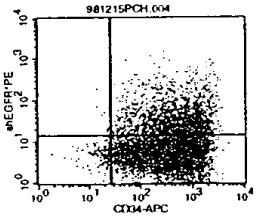


File: 981215PCH.003
Sample ID: CD34+/pG1αhEGFR-IN/Isotypes
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

Quad	% Gated	X Mean	Y Mean
UL	30.58	8.18	63.50
UR	0.14	32.20	152.92
LL	68.89	7.72	6.35
LR	0.38	520.32	4.13

α EGFR PE
ISO APC

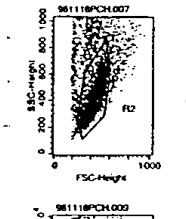


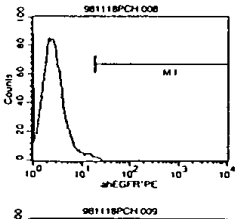


File: 981215PCH.004
Sample ID: CD34+/pG1αhEGFR-IN/αCD34*AP
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

Quad	% Gated	X Mean	Y Mean
UL	0.35	19.59	95.02
UR	27.03	772.56	64.13
LL	2.25	18.25	5.79
LR	70.37	601.24	5.88

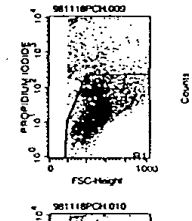
α EGFR
CD34

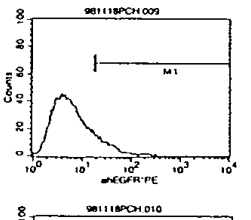




File: 981118PCH.008
Sample ID: PBL/Mock
Gate: G3
X Parameter: FL2-H αhEGFR*PE (Log)

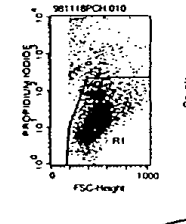
Marker	% Gated	Mean	Median
All	100.00	3.07	2.48
M1	0.35	21.13	20.17

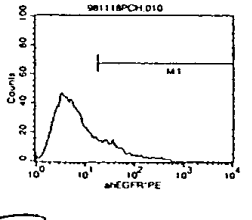




File: 981118PCH.009
Sample ID: PBL/79
Gate: G3
X Parameter: FL2-H αhEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	11.11	5.47
M1	12.99	44.98	30.92





File: 981118PCH.010
Sample ID: PBL/81
Gate: G3
X Parameter: FL2-H αhEGFR*PE (Log)

Marker	% Gated	Mean	Median
All	100.00	18.57	5.76
M1	19.76	70.15	38.72

Continued on Page 21

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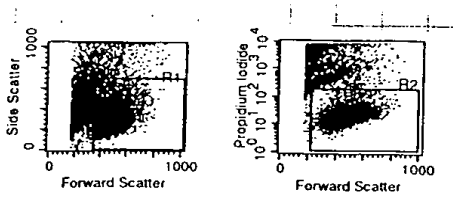
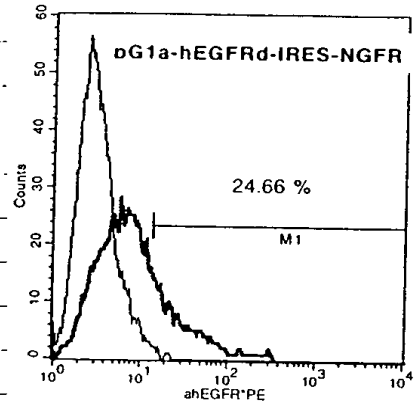
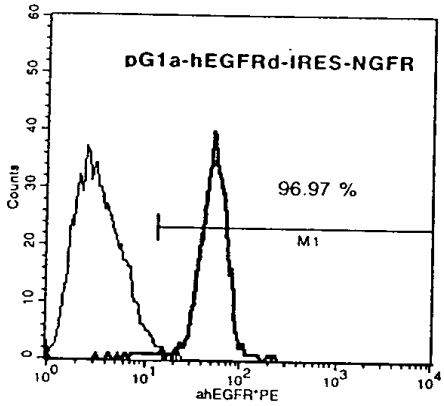
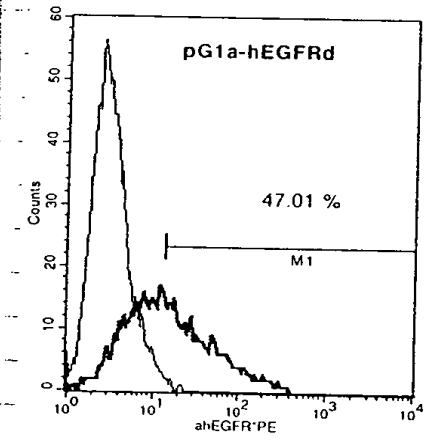


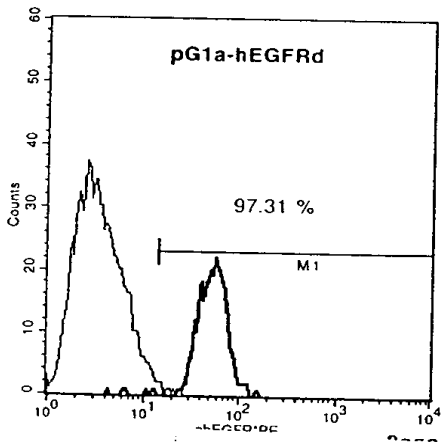
Before Bead Selection

After Bead Selection









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SYSTEMIX	
Order Number:	033670 01
Order Date:	[redacted]

GIBCO BRL Custom Primers

Certificate of Analysis

Primer 1:

Primer Name: EGFR1
Researcher: Susanne Pippig
Sequence (5' to 3'): CTA GGC TAG CAT GCG ACC CTC CGG GAC GGC C

Molecular Weight (µg/µmole):	9993.2	Primer Number:	M0371C12 (C12)
Millimolar Extinction Coeff.:(OD/µmol)	320.5	Primer Length:	31
		Scale of Synthesis:	50nmol
Purity	**Standard**	µg per OD:	31.1
Tm (1 M Na+)	**89**	nmoles per OD:	3.1
Tm (50 mM Na+)	**67**	**OD's**	**31.35**
% GC	**70**	**µg's***	**977.59**
Notes:		**nmoles**	**97.8**
		Coupling Eff.	**99%**

Primer 2:

Primer Name: EGFR2
Researcher: Susanne Pippig
Sequence (5' to 3'): CTC TGC CCG GCG AGT CGG GCT GAC AGC TAT GAG ATG GAG GAA

Molecular Weight (µg/µmole):	13742.4	Primer Number:	M0371D01 (D01)
Millimolar Extinction Coeff.:(OD/µmol)	465.6	Primer Length:	42
		Scale of Synthesis:	50nmol
Purity	**Standard**	µg per OD:	29.5
Tm (1 M Na+)	**91**	nmoles per OD:	2.1
Tm (50 mM Na+)	**69**	**OD's**	**30.09**
% GC	**61**	**µg's***	**887.97**
Notes:		**nmoles**	**64.6**
		Coupling Eff.	**99%**

Primer 3:

Primer Name: EGFR3
Researcher: Susanne Pippig
Sequence (5' to 3'): TTC CTC CAT CTC ATA GCT GTC AGC CCG ACT CGC CGG GCA GAG

Molecular Weight (µg/µmole):	13484.4	Primer Number:	M0371D02 (D02)
Millimolar Extinction Coeff.:(OD/µmol)	427.2	Primer Length:	42
		Scale of Synthesis:	50nmol
Purity	**Standard**	µg per OD:	31.5
Tm (1 M Na+)	**91**	nmoles per OD:	2.3
Tm (50 mM Na+)	**69**	**OD's**	**16.66**
% GC	**61**	**µg's***	**525.99**
Notes:		**nmoles**	**38.9**
		Coupling Eff.	**99%**

FOR LABORATORY RESEARCH USE ONLY.

CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

* -See Note about Quantities in Supporting Information.

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SYSTEMIX
Order Number: 033670 02
Order Date:

GIBCO BRL Custom Primers

Certificate of Analysis

Primer 1:

Primer Name: EGFR# 14
Researcher: Susanne Pippig
Sequence (5' to 3'): GTT CCT GTG GAT CCA GAG GAG

Molecular Weight (µg/µmole):	6842.2
Millimolar Extinction Coeff.:(OD/µmol)	231.7

Primer Number:	Z7143C02 (C02)
Primer Length:	21
Scale of Synthesis:	50nmol
µg per OD:	29.5
nmoles per OD:	4.3

Purity	**Standard**	**OD's**	**10.80**
Tm (1 M Na+)	**73**	**µg's***	**319.05**
Tm (50 mM Na+)	**51**	**nmoles**	**46.6**
% GC	**57**	**Coupling Eff.**	**99%**

Notes:

Primer 2:

Primer Name: GABA2
Researcher: Susanne Pippig
Sequence (5' to 3'): GGT TCA AGA TCT ACG ACC CTT

Molecular Weight (µg/µmole):	6721.2
Millimolar Extinction Coeff.:(OD/µmol)	223.9

Primer Number:	Z7143C03 (C03)
Primer Length:	21
Scale of Synthesis:	50nmol
µg per OD:	30.0
nmoles per OD:	4.4

Purity	**Standard**	**OD's**	**9.44**
Tm (1 M Na+)	**69**	**µg's***	**283.50**
Tm (50 mM Na+)	**47**	**nmoles**	**42.2**
% GC	**47**	**Coupling Eff.**	**98%**

Notes:

Primer 3:

Primer Name: GABA5
Researcher: Susanne Pippig
Sequence (5' to 3'): CCC TCA CTT ATA AAG CAA ATG

Molecular Weight (µg/µmole):	6698.2
Millimolar Extinction Coeff.:(OD/µmol)	236.9

Primer Number:	Z7143C04 (C04)
Primer Length:	21
Scale of Synthesis:	50nmol
µg per OD:	28.2
nmoles per OD:	4.2

Purity	**Standard**	**OD's**	**10.99**
Tm (1 M Na+)	**65**	**µg's***	**310.76**
Tm (50 mM Na+)	**43**	**nmoles**	**46.3**
% GC	**38**	**Coupling Eff.**	**98%**

Notes:

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CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

* -See Note about Quantities in Supporting Information.

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Set cytokine prod.
for prolif
+ PHA + feeder + 40u-3R+2
+ 20u 3R-2 / ml
+ nothing

PCR to generate deletion in the XC domain for the EGFR

(21)
- V 1ℓ EGFR plasmid (10ng/ℓ)
- V 1ℓ EGFR 1
- V 1ℓ EGFR 3
- V 2ℓ dNTPs
- V 10ℓ Pfu buffer
- 1ℓ Pfu
- V 84ℓ H_2O

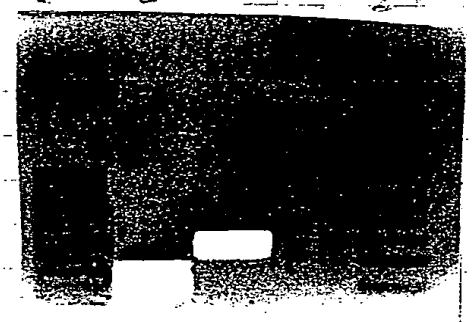
100ℓ

(22)
- V 1ℓ EGFR pl
- ✓ ℓ EGFR 2
- V 1ℓ 4
- V "
- ✓ "
- ✓ "
- ✓ "

100ℓ

30 cycles @ 55°C

V 21 V V



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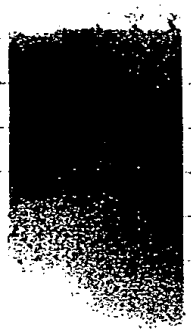
Hyclone
13/14 21/22

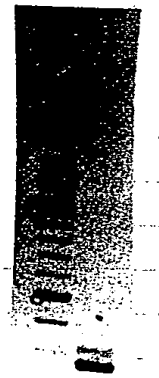


-233
-230-30

#23 ✓ 1µl EGFR1
1µl " 4
✓ 10µl 21
✓ 5µl 22
✓ 2µl dNTPs
✓ 10µl Pfu buffer
✓ 70µl H_2O
1µl Pfu
100µl

30 cycles @ 55°

Add thymidine to proliferation assay.



result from PCR #23

Hyclone 13/14 | PCR 22/23

- upper band is probably the correct product, cut out and reamplify

Continued on Page

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Maria Farrell Signed [redacted] Date

Day 3 staining T68515 for CD25

1) CD4 + iso TC
2) + CD25 TC

745

1171

1190

10) 1200

} UGFR FITC
CD4 PE

New buffycoat V15721

cell # 1) 156 x 2 x 50 = 15600 x 10^4 = 1.5×10^8
2) 124 x = 1.24×10^8

150 µl α-CD4 bio per tube
500 µl beads -> activate

PCR # 24: ✓ 10 µl PCR 23 upper band
✓ 1 µl EGFR1
✓ 1 µl 4
✓ 10 µl Pfu buffer
✓ 2 µl dNTPs
1 µl Pfu
✓ 75 µl H_2O
100 µl

30 cycles @ 60°C

Continued on Page

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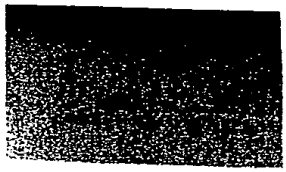
strategy
#24 PCR prod.

Cut upper band
-> gel isolate

after gel extraction

2 µl -> 500 / 10-µ



PCR # should be 309 b

Cut with BamHI / NheI in buffer H
and pCineo EGFR Δ
use all of gel elution

28 µl reaction
4 buffer H V
2µl BamHI
2µl NheI 4µl H2O V
40µl @ 37°C o/n

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PCR #24

Continue cloning of EGFR ΔXC into pCineo EGFR ΔC

1) ✓ 3 µl plasmid (= 3 µg)
✓ 1 µl Nhe I
1 µl Bam HI
✓ 2 buffer H
✓ 13 µl H_2O
20 µl

CIP same as #3

Cloning of Flag/Gcba into pCineo Gcba

2) ✓ 3 µl Flag/Gcba (#104)
1 µl Nhe I
✓ 1 µl buffer H
✓ 5 µl H_2O
10 µl

2 hrs @ 37°C

✓ + 1.5 µl buffer H
1 µl PstI
✓ 7.5 µl H_2O
20 µl

3) ✓ 1 µl pCineo Gcba
1 µl Nhe I
✓ 1 µl buffer H
✓ 8 µl H_2O
10 µl

✓ "
"
✓ "
20

+ 8 µl CIP buffer
6 µl CIP
44 µl H_2O

Continued on Page

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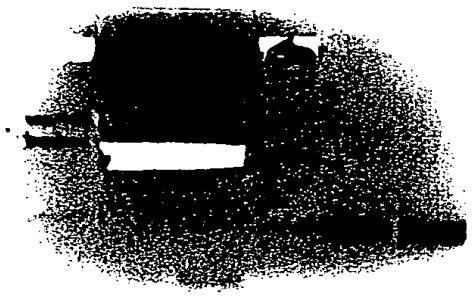
PCR repeat PCR #24 ✓✓ 10ℓ #23
1ℓ EGFR1
✓ ✓ 1ℓ EGFR4
✓ ✓ 10ℓ Phu buffer @ 62°C
✓ ✓ 2ℓ dNTPs 30 cycles
1ℓ Phu
✓ ✓ 75ℓ H2O
100

#25 ✓ 20ℓ #21
✓ 5ℓ #22
1ℓ EGFR1
✓ 1ℓ
✓ 10ℓ Phu b
✓ 2ℓ dNTPs
1ℓ Phu
✓ 60ℓ H2O
100

1 2 3 4 24 25



against a doubled
shows up

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Cut PCR 24 and 25 with NheI | BamHI [redacted]

40ℓ #24	40ℓ #25
5ℓ buffer H	5ℓ buffer H
2.5ℓ NheI	2.5ℓ NheI
2.5ℓ BamHI	2.5ℓ BamHI
50ℓ	50ℓ

Blunt BamHI site in pCRScript that contains EGFRΔIC [redacted]

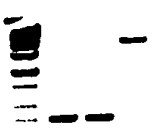
1ℓ pCRScript EGFRΔIC
2ℓ buffer B
2ℓ BamHI
15ℓ H_2O
20ℓ

1hr @ 37°C

Reamplify #24 and clone blunt end into pCR Script @ 62°C ->#26

gel isolate 2, 3 and 4

Single digest
5ℓ 1 1 1



1) ✓2ℓ #2 2) ✓2ℓ #3 3)
2ℓ #4 2ℓ #4 2ℓ #4
✓4ℓ H_2O ✓4ℓ H_2O ✓6ℓ H_2O
2ℓ 5x buffer 5x buffer 2ℓ 5x buffer

10ℓ
+10ℓ 2x buffer
-2ℓ Enzyme

didn't get any clones =(Continued on Page 8

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B. Pippy [redacted]
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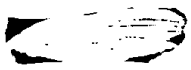
Single colonies

10 µg — — #26 ~ 40 - 50 µg



Ligations

I ✓ 1 µl PCR Script
✓ 1.5 µl #26 (EGFR xCA)
✓ 1 µl PCR Script reaction buffer
✓ 0.5 µl ATP
✓ 4 µl H_2O
1 µl Sal I
1 µl T4 DNA ligase
10 µl

II ✓ 1 µl CIP
✓ 3 µl #2
✓ 4 µl H_2O
2 µl 5× buffer
10 µl 2× buffer
1 µl ligase

III ✓ 1 µl CIP
3 µl #3
✓ 4 "
" "
" "
" "

IV ✓ 1 µl CIP
✓ 7 µl H_2O
" "
" "
" "

V ✓ 1 µl SAP
✓ 3 µl #2
✓ 4 µl H_2O
2 µl 5× buffer
10 µl 2 × b
1 µl ligas

VI ✓ 1 µl SAP
3 µl #3
✓ "
" "
" "
" "

VII ✓ 1 µl SAP
✓ 7 "
" "
" "
" "

Continued on Page

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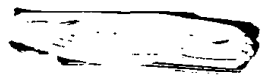
-> still didn't get any colonies after transformation



use less ligation, instead of 5 (for I) or 10l (I-VII) only 2l (works better according to Ross)

retransform #I, V, VII

-> also cut pCiteo Gebak and mini preps 7 and 8 again with NheI and PstI, ligate, but no clipping or zapping

1) ✓ 2l pCiteo Gebak
✓ 2l buffer H
2l NheI
✓ 14l H2O
20l

2) ✓ 5l mini #7
✓ 2l buffer H
" "
✓ II

3) ✓ 5µl mini #2
✓ "
" "
✓ II

2hrs @ 37°C

+ 3l buffer H
2l PstI
15l H2O
40l

run and cut out bands

Continued on Page

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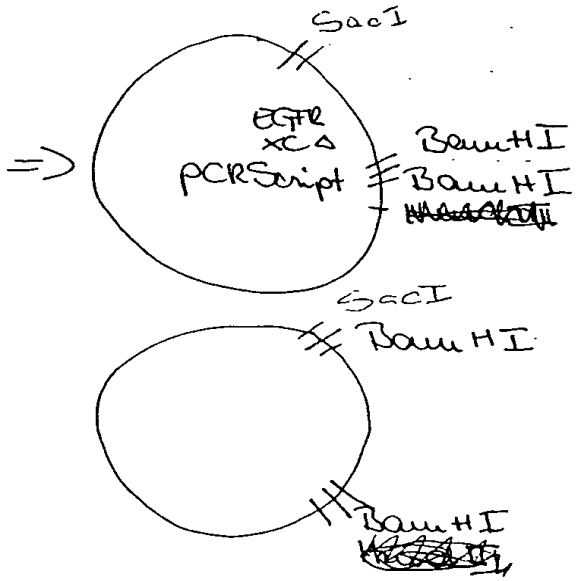
=> got about 200 clones per ligation #1 after the transformation
=> less ligation seems to work better

Let Peter check minis with Bam HI alone and Bam HI + ~~Hind III~~ Sac I



Bam HI ~~350~~ vector linearized
+ ~~Hind III~~ Sac I 350 + 3 kb

Bam HI 350 + 3 kb
B + ~~B~~ "

Set up new ELISA for JFN of day 1:

20 µl as starting point: ~~240 µl dilution buffer + 60 µl~~

400 µl dil. b
+ 100 µl sup. ⟩ 250
250 dil. b. ⟩ 4 dilutions

set up plate ~~same as for 7E-1 etc~~
see next page

Continued on Page

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S. Ryff
Signed

[redacted]
Date

Signed

[redacted]
Date

Test various α-EGFR antibodies [redacted]

Transfect 293T with wt EGFR(100), EGFRΔIC(81), EGFRΔXCΔIC(121)

- seed 8×10^6 cells the day before transfection

100 : 1.18 µg	12.7 µl	+ 75 µl $CaCl_2$	+ 512 H_2O	= 600
81 : 1 µg/µl	=> 15 µl		510	= 600
121 : 1.15 µg	13 µl		512	= "

+ 600 2× HBS

according to Janet's protocol

Isolation DNA from Pisclia clones

followed Invitrogen protocol

- used lyticase instead of Zymolyase, didn't have enough units and incubated the mixture for 1hr @ 37°C instead of 30°C
  => then left mixture o/n at 30°C

To make probe:

10 µg	pPICZαB MuSK #30
≈ 10 µl	
2.5 µl	EcoRI
2.5 µl	ClaI
5 µl	buffer H
30 µl	H_2O
50 µl	

Continued on Page

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finished prep. of yeast DNA [redacted]

thaw Cos-1 cells

New transfection of 293 cells [redacted]

- this I pelted ~ 3×10^6 day before transfection
- transfection itself was done as an [redacted]

mock: 131 $\times 20 \times 10^4 = 2620 \times 10^4 = 2.6 \times 10^7$ 350 µl
100: 126
81: 123
121: 184

staining:

1)	#100	PI	
2)	#100	Jg2a	
3)	#100	Jg2b	
4)	#100	EGFR-PE	
5		αEGFRPE	1
6	mock	GR01	2
7	mock	GR05	3
8			1
9	100		2
10	100		3
11			1
12	81		2
13	81		3
14			1
15	121		2
16	121		3

FACS # are different

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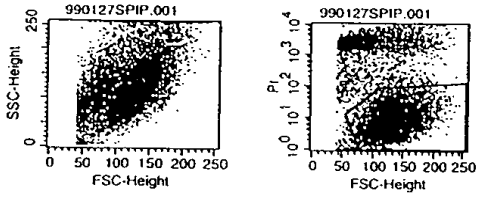
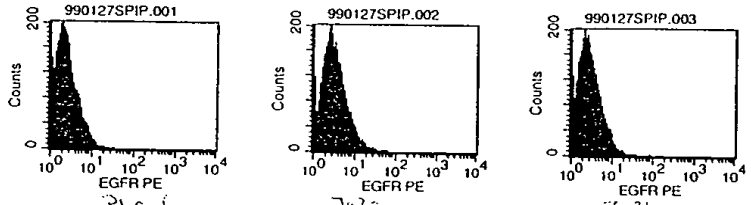
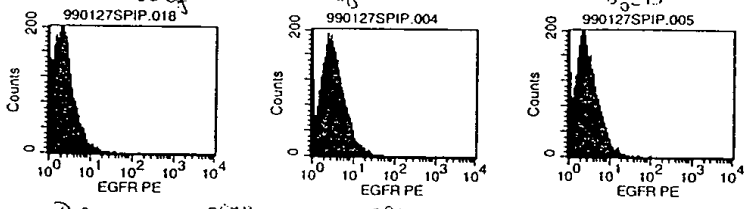
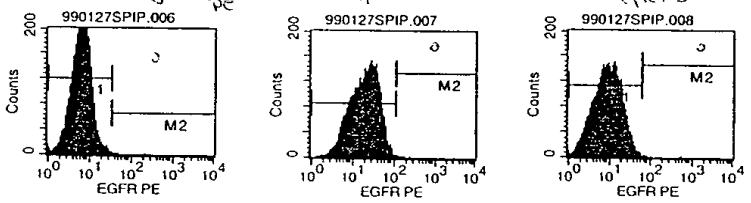
[redacted] Signed [redacted] Date



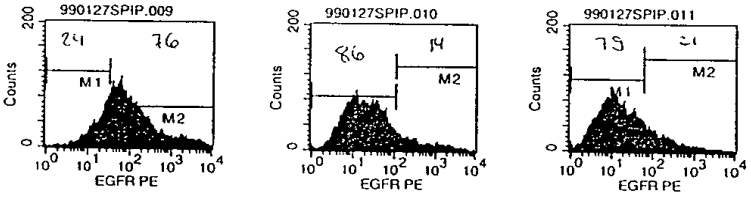
results from p. 17



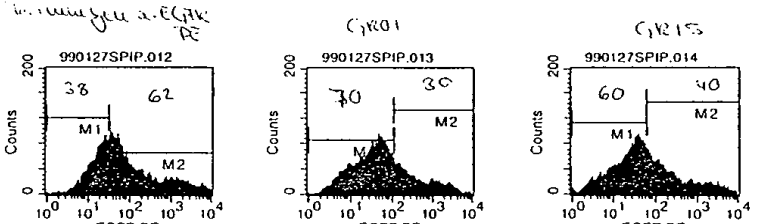




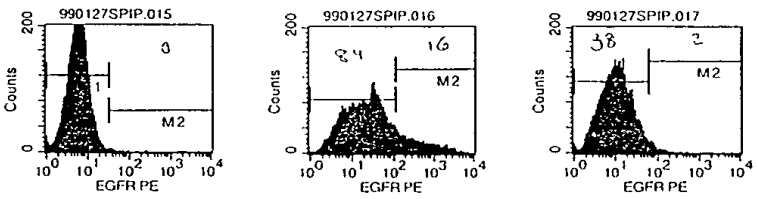
mock



w EGFR #100



#151 EGFR mAb



#121 EGFR mAb

Continued on Page

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Purpose clone hEGFR ΔEC

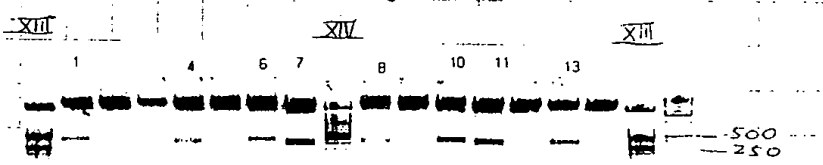
A. pCRScript-hEGFRΔEC #118

Suganne has lgt hEGFR ΔEC into pCRScript Need to check w/ BamHI + Sac I

[redacted] innoculate 3 clones from [redacted] & 12 clones from [redacted] (#4-14) → 3 mL Bio 101 RPM mini-prep w/ 2 mL O/N cultures

	mini
mini	8
A	2
BamHI (10U/λ)	1.75
Sac I (40U/λ)	0.25
dd H_2O	8
	20 λ

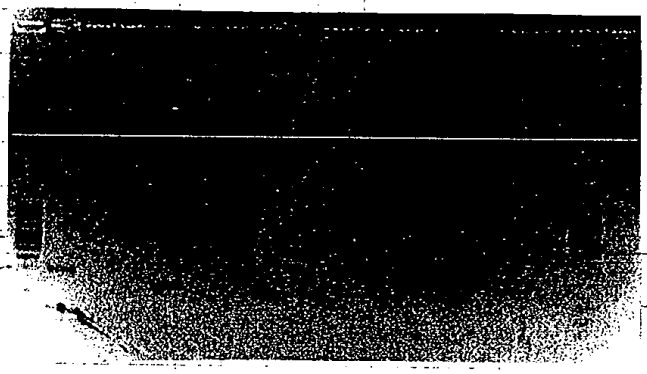
37°C 2 hrs



↓ run longer

2% TEA agarose

← gel "smiling"



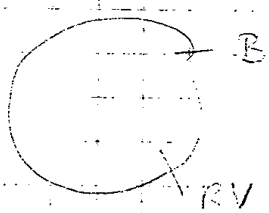
#1, 4, 6, 7, 8, 10, 11, 13 show expected fragment sizes of 350, 3000 bp

Innoculate #1 (≡ 118.1) for maxi
6 (≡ 118.6)

Send 118.1 & 118.6 for sequencing

B. pG1a-hEGFRΔEC

pG1a



Not + Xho I

→ Sequence [redacted]

See pg 11 for RE analysis & td & FACS

Continued on Page 8

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Peter Chang [redacted] [redacted] [redacted]

Signed Date Signed Date

c. To clone hEGFRΔEC into hEGFRΔ

#81	
pGka-hEGFRΔ	20
B	5
BamHI	5
ddH2O	20
	50

37°C 3hrs
expect 885 5880 bp

#119.1	
pGla-hEGFRΔ/C	20
B	5
BamHI	5
ddH2O	20
	50

37°C 3hr
expect 18, 5005 bp

#83	
pCRSα-hEGFR	20
BamHI	5
EcoRS	5
B	10
ddH2O	60
	100

37°C 3hrs
expect 2127, 2976

#118.1
pCRSα-hEGFR
Bam-I
RS
B
dd-O
37°C 3
expect 18.

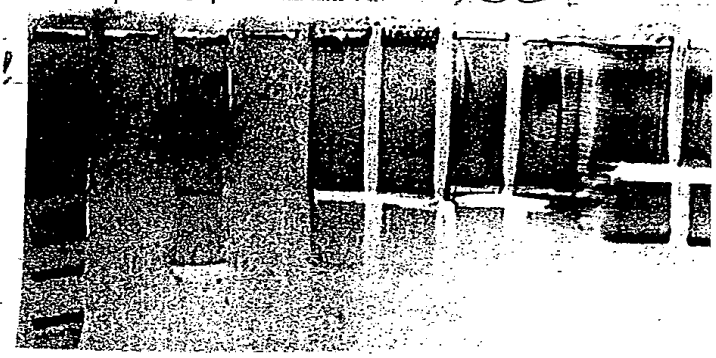
dephos #119.1 & #118.1 ⇒ 50λ RE
10λ CIP buffer
10λ CIP
30λ
100 37°C 2hr

gel isolate

BamHI +RS 83 | BamHI +RS 1181.1 | BamH 1191.

BamHI 81

#83 seems weird ∴ abandon cloning pCRSα-hEGFRΔ into pCRSα-hEGFRΔEC!

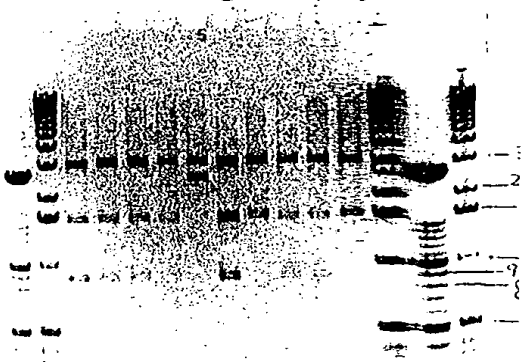


(λ)

lig.	I	V
119 Φ	1	0
119+81 (½)	1	4
119+81	1	7

25°C ~~20°C~~ 20'
+fm XL10 gold
leave on bench at 25°C over the weekend.

pGla-hEGFRΔEC-hEGFRΔ
1 2 3 4 5 6 7 8 9 10



Innoculate

Mini 4
B 1
EcoRS 1
ddH2O 4
10λ
37°C 1hr
load all

correct orientation	opposite	No insert
75	75	75
76	76	76
231	231	231
3016	3016	3016
32	917	75
2460 bp	1575 bp	
#5	#1-4, 7-10	none

1% TEA 1hr @ =>✓

Continued Page

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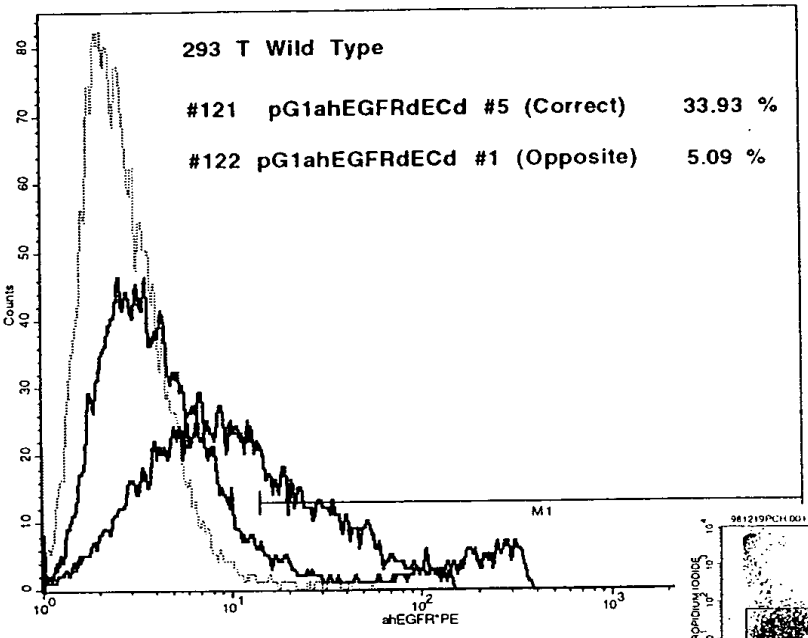
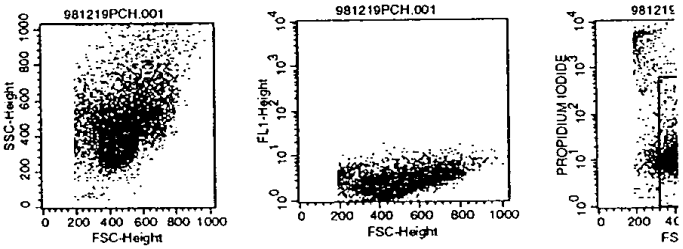
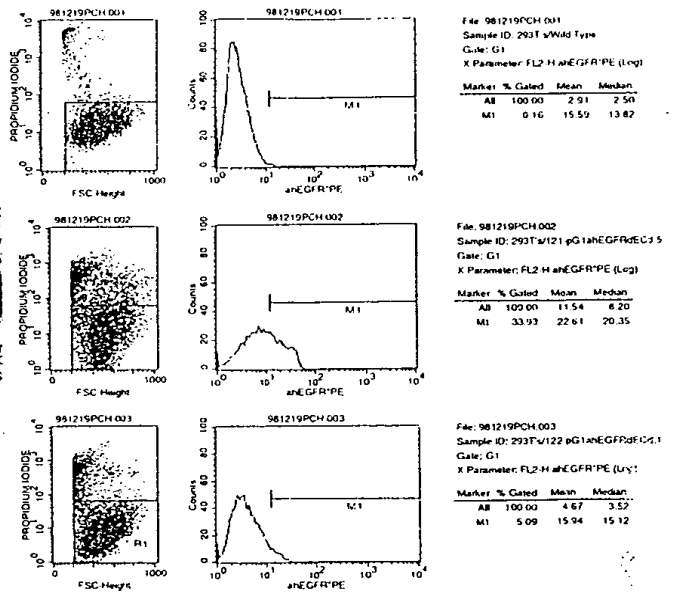
Innoculate #5 & 1 for maxipsep
Seed 293 T's for transfection
Transfect 30 µg DNA
15 µg pC1GL
15 µg pC1GP
74 λ $CaCl_2$
→ 600 λ w/ Clontech's CaPO4 kit
add drop by drop 600 λ HEPES
DI H_2O while vortexing
25°C 25'
Add 50 λ 10 mM chloroquine to 10 mL in 100 mm dish
Add 1.2 mL mixture drop by drop
37°C 8 PM ~

Overlay of 293T's Transfected with human EGFR Extracellular Deletion Mutant







- Change fresh media ~ 9AM.
- FACS ~ 10 AM ∴ 36 hrs post-transfection
- collect sup's at 36 & 60 hrs

Future action:
① Td PPA6's
② Sort PPA6's

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